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Phylogenic Relationship of *Allium* Species in Subgenus *Rhizirideum* by PCR DNA Fingerprint

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ABSTRACT: *Allium* is one of the largest genera, which has more than 700 species. PCR by URP (universal rice primer) primers was carried out to get phylogenetic information on 26 species, 62 accessions of subgenus *Rhizirideum*. The accessions were divided into seven groups at 0.76 similarity level. *A. tuberosum* (Chinese chives) and *A. ramosum* represented high similarity of 0.91. *A. montanum*, *A. nutans*, *A. senescens*, *A. libani*, *A. odorum*, *A. austrosibiricum*, and *A. narcissiflorum* grouped at 0.80 similarity. Some of the wild species, such as *A. prostratum*, *A. polyrhizum*, *A. odorum*, and *A. mongolicum*, showed different band patterns according to polyploidy, occurrence of B-chromosome, collection site, and origin.

Keywords : *Allium*, *Rhizirideum*, Chinese chives, PCR, and URP primer

The genus *Allium* comprises 750 species within six subgenera, 46 sections and 11 subsections, and is distributed in huge geographical and ecological areas (Hanelt *et al.*, 1992; Stearn, 1992). Subgenus *Rhizirideum*, one of the two largest subgenera in *Allium*, is a heterogeneous group. Section *Rhizirideum* which are mainly distributed in Eurasian steppe zone still needs further taxonomic revision (Hanelt, 1990). Morphological traits for typical distinction of section *Rhizirideum* from section *Cepa* or section *Schoenoprasum* is its floret and leaves. In section *Rhizirideum*, leaves are flat and florets are usually cup-shaped or bell-shaped, rarely star-shaped or almost globular. In Mongolia 46 species of *Allium* was reported (Sancir, 1992).

Identification of closely related species is highly recommended to introduce useful characters from wild relatives to cultivated *Allium*, due to its hybridization barrier (Valk *et al.*, 1991; Khrustaleva, 1998). *A. roylei* had been grouped in section *Rhizirideum*, but it was rearranged in section *Cepa*. Its resistance to downy mildew, one of the most serious diseases in onion cultivation, was successfully introduced into *A. cepa* (Kofoet *et al.*, 1990). Chinese chives have been adapted both in tropical and temperate climates (Kamenetsky, 1993). Since the plants keep green until temperatures fall below 4~5°C, it considered as an all year round vegetable in warm region.

Few reports are available on phylogenic relationship of section *Rhizirideum* on both PCR and RFLP (Dubouzet *et al.*, 1997), compared to reports on morphological and cytological data. PCR DNA fingerprint by URP primers was accomplished to analyze phylogenic relationship of 26 species, 62 accessions of *Allium* L. subgenus *Rhizirideum*. URP primers were designed from repeated sequences of Korean wild rice (Kang *et al.*, 1997). It has been successfully applied in inter-specific classification of fungi, bacteria and fishes as well as plants. The main difference of URP-PCR from RAPD or AP-PCR (arbitrarily primed-polymerase chain reaction) is the use of relatively long (20 bp) primers, designed for fingerprinting any organism at a relatively high annealing temperature. Thus, high PCR reproducibility is expected (Wu *et al.*, 1991).

MATERIALS AND METHODS

Plant materials and DNA extraction

Twenty-six species, 62 accessions of *Allium* L. subgenus *Rhizirideum* were used for this study (Table 1). Leaves from three plants of each accession that was over wintered in plastic houses in Suwon were collected for DNA extraction. Genomic DNA was extracted by Plant Mini DNeasy kit (Qiagen, Germany).

PCR and data analysis

Primers and PCR conditions are adopted from Kang *et al.* (1997). Sequences of URP (Universal Rice Primer) primers are listed in Table 2. Amplification of *Allium* genomic DNA using URP primers was performed in a PTC-100 thermal cycler (MJ Research, USA) programmed for 4 min at 94°C and 36 cycles of 1 min at 94°C, 2 min at 55°C, and 2 min at 72°C. PCR was carried out in 50 μl of reaction solution composed of 70-100 ng template DNA, 4 μl dNTP (2.5 mM), 5 μl 10X buffer (100 mM Tris-HCl, pH8.3, 500 mM

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Table 1. List of accessions of *Allium* L. subgenus *Rhizirideum* (G. Don ex Koch) Wendelbo.

No.	Scientific name	Collection site, Origin or Variety/Common name	Chromosome No. (2n) †	Section‡
1	*A. ansiopodium* Ledeb Khobd	S. Govi, MNG	32	*Anisopodia* Ined.
2	*A. ansiopodium* Ldbeb Khobd	Khobd, MNG	16(1B)	*Anisopodia* Ined.
3	*A. austrosibiricum* N. Fries.	RUS	ND¶	*Rhizirideum* G. Don
4	*A. lineare* L.	Dornod, MNG	32	*Reticulato-bulbosa* R. Kam.
5	*A. lineare* L.	Bulgan, MNG	32	*Reticulato-bulbosa* R. Kam.
6	*A. lineare* L.	Tob, MNG	32	*Reticulato-bulbosa* R. Kam.
7	*A. lineare* L.	Bulgan, MNG	32	*Reticulato-bulbosa* R. Kam.
8	*A. libani* Boiss.	RUS	ND	*Rhizirideum* G. Don
9	*A. senescens* ssp. *montanum* Holub	RUS	32	*Rhizirideum* G. Don
10	*A. senescens* ssp. *montanum* Holub	AUT	32	*Rhizirideum* G. Don
11	*A. nutans* L.	RUS	32	*Rhizirideum* G. Don ex Koch
12	*A. nutans* L.	RUS	32	*Rhizirideum* G. Don ex Koch
13	*A. odorum* L	Bulgan, MNG	32	*Rhizirideum* G. Don
14	*A. odorum* L.	Songino, MNG	32(34)	*Rhizirideum* G. Don
15	*A. odorum* L.	Selenge, MNG	16+2B	*Rhizirideum* G. Don
16	*A. prostratum* Trev.	Darkhan, MNG	16(1B)	*Rhizirideum* G. Don
17	*A. prostratum* Trev.	Songogor, MNG	32	*Rhizirideum* G. Don
18	*A. prostratum* Trev.	Bulgan, MNG	16+1B	*Rhizirideum* G. Don
19	*A. prostratum* Trev.	RUS	16	*Rhizirideum* G. Don
20	*A. ramosum* L.	SUN	32	*Butomissa* R. Kam.
21	*A. senescens* L.	MNG	40	*Rhizirideum* G. Don
22	*A. senescens* L.	Tob, MNG	40	*Rhizirideum* G. Don
23	*A. cyaneum* Regel	Halla Mt., JeJu, KOR	16	*Rhizirideum* G. Don
24	*A. senescens* L.	Ulleungdobuchu	32	*Rhizirideum* G. Don
25	*A. tuberosum* Rottl. ex Spreng.	Baekseokbuchu	32	*Butomissa* R. Kamelin
26	*A. tuberosum* Rottl. ex Spreng.	Cheongnimbuchu	32	*Butomissa* R. Kamelin
27	*A. senescens* L.	Pabuchu	32	*Rhizirideum* G. Don
28	*A. senescens* var. *minor*	Solipbuchu	16	*Rhizirideum* G. Don
29	*A. tuberosum* Rottl. ex Spreng.	Muju, KOR	32	*Butomissa* R. Kamelin
30	*A. tuberosum* Rottl. ex Spreng.	Pyeongchang, KOR	32	*Butomissa* R. Kamelin
31	*A. tuberosum* Rottl. ex Spreng.	Jeongseon, KOR	32	*Butomissa* R. Kamelin
32	*A. tuberosum* Rottl. ex Spreng.	Ulju, KOR	32	*Butomissa* R. Kamelin
33	*A. tuberosum* Rottl. ex Spreng.	Yesan, KOR	32	*Butomissa* R. Kamelin
34	*A. tuberosum* Rottl. ex Spreng.	Kochang, KOR	32	*Butomissa* R. Kamelin
35	*A. tuberosum* Rottl. ex Spreng.	WanJu, KOR	32	*Butomissa* R. Kamelin
36	*A. tuberosum* Rottl. ex Spreng.	TWN	32	*Butomissa* R. Kamelin
37	*A. tuberosum* Rottl. ex Spreng.	CHN	32	*Butomissa* R. Kamelin
38	*A. ramosum* L.	SUN	32	*Butomissa* R. Kamelin
39	*A. bidentatum* Fisch. ex Prokh.	MNG	32	*Caespitosoprason* Friesen
40	*A. mongolicum* Regel	MNG	16	*Caespitosoprason* Friesen
41	*A. mongolicum* Regel	Ubs, MNG	16	*Caespitosoprason* Friesen
42	*A. mongolicum* Regel	Khobd, MNG	32	*Caespitosoprason* Friesen
43	*A. mongolicum* Regel	M. Govi, MNG	16	*Caespitosoprason* Friesen
44	*A. mongolicum* Regel	M. Govi, MNG	32	*Caespitosoprason* Friesen
45	*A. polyrhizum* Turcz. ex Regel	MNG	ND	*Caespitosoprason* Friesen
46	*A. polyrhizum* Turcz. ex Regel	S. Govi, MNG	32	*Caespitosoprason* Friesen
47	*A. condensatum* Turcz.	MNG	16	*Oreiprason* F. Herm.
48	*A. hymenorrhizum* Ledeb.	MNG	16	*Oreiprason* F. Herm.
49	*A. hymenorrhizum* Ledeb.	CAN	16	*Oreiprason* F. Herm.
50	*A. hymenorrhizum* Ledeb.	RUS	ND	*Oreiprason* F. Herm.
51	*A. obliquum* L.	MNG	16	*Petroprason* F. Herm.
52	*A. obliquum* L.	Bayan olgil, MNG	48	*Petroprason* F. Herm.
53	*A. obliquum* L.	RUS	16	*Petroprason* F. Herm.
54	*A. amphibolum*	RUS	32	*Rhizirideum* G. Don

Table 1. Continued.

No.	Scientific name	Collection site, Origin or Variety/Common name	Chromosome No. (2n)†	Section‡
55	*A. eduardii* Stearn.	Selenge, MNG	16	*Reticulato-bulbosa* R. Kam.
56	*A. eduardii* Stearn.	S. Govi, MNG	16(1B)	*Reticulato-bulbosa* R. Kam.
57	*A. leucocephalum* Turcz. ex Ldb.	Baruuncharea, MNG	16	*Reticulato-bulbosa* R. Kam.
58	*A. leucocephalum* Tercz. ex Ldb.	S. Govi, MNG	16	*Reticulato-bulbosa* R. Kam.
59	*A. narcissiflorum* Villars	RUS	ND	*Narkissoprason* Herm§
60	*A. macrostemon* Bunge	MNG	32	*Haplostemon* Boiss.
61	*A. splendens* Willd ex Schult	Gorchi, MNG	48	*Reticulato-bulbosa* R. Kam.
62	*A. thunbergii* G. Don	PyeongChang, KOR	32	*Sacculiferum* P. Gritz.

†Buyanchimeg (1997) and the authors; ‡Chinese Academy of Science (1980), Pritsch (1992), Sancir (1992), Hanelt (1996), and Ohri *et al.* (1998); §subgenus *Amerallium* Traub.
¶ND: Not determined.

Table 2. List of URP Primers used in PCR.

URP Primer	Sequence (5' to 3')	Tm (°C)
2R	CCCAGCAACTGATCGCACAC	67.3
4R	AGGACTCGATAACAGGCTCC	66.3
9F	ATGTGTGCGATCAGTTGCTG	62.7
13R	TACATCGCAAGTGACACAGG	62.6

KCl, 20 mM $MgCl_2$, 0.1% gelatin (Sigma)), 2 μl URP primer (100 ng), and 2.5 unit of DNA polymerase with combination ratio of 30 *Taq* (Takara Shuzo, Kyoto) and 1 native *Pfu* (Stratagen Co.). Amplified DNA products were electrophoresed on 1.5% agarose gel on 70-90V for 7 hours and followed by staining on EtBr. Individual URP-PCR products of each *Rhizirideum* accession were scored for their presence (value=1) or absence (value=0). The phylogenic analysis was done by Nei method (Nei, 1987). The similarity coefficient (F) was calculated as the fraction of shared fragments between pairs of the accessions. For accessions, x and y, F=2Nxy/(Nx+Ny), where Nxy is the number of DNA fragments shared by accessions X and Y, while Nx and Ny are the number of fragments scored from the accessions X and Y, respectively. On the basis of the similarity coefficient, a dendrogram was constructed with the statistical program NTSYSpc (version 2.0, Exter Software, Setauket, NY) using the unweighted pair-group method with arithmetic mean (UPGMA).

RESULTS AND DISCUSSION

PCR fingerprinting of *Allium* subgenus *Rhizirideum*

DNA amplification by four URP primers which were pre-screened from 12 primers was performed from 26 species, 62 accessions of subgenus *Rhizirideum*. A high stringent PCR was employed in annealing step to give PCR specificity between template DNA and primer.

Totally 148 polymorphic bands varying in size from 100 bp to 4,000 bp were produced from pre-selected URP-2R,

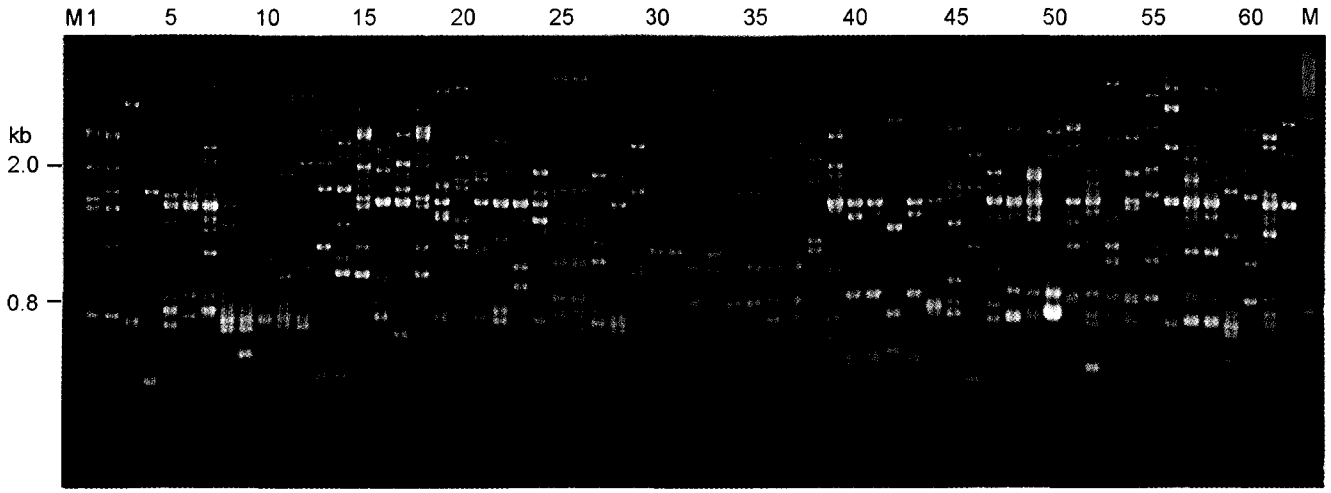

Fig. 1. DNA profiles from 62 accessions of *Allium* L. subgenus *Rhizirideum* with URP-4R primer.

4R, 9F and 13R primer. Combination of *Taq* polymerase and native *Pfu* polymerase amplified high density of large sized bands (more than 1.5 kb) when compared to *Taq* polymerase alone (Dubouzet *et al.*, 1996). From the pre-screen for optimal combination ratio from 1 : 1 to 50 : 1, combination ratio of 30 *Taq* and 1 *Pfu* produced optimal results (Data not shown). Each URP-PCR profile that was produced by our PCR protocol allowed to discrimination of *Rhizirideum* accessions at intra- and inter-species levels. The genetic similarity index calculated from the PCR fingerprinting bands, which were amplified by URP primers, was used to estimate the phylogenic relationship among *Rhizirideum* accessions. Based on the URP-PCR fingerprint data, the genetic distance was used to construct a dendrogram for the *Rhizirideum* accessions analyzed. Fig. 2 shows the genetic relationships of the accessions on the basis of URP-PCR data. *Rhizirideum* accessions were clustered into seven large groups corresponding to their genomic patterns and an average similarity value of them was revealed as 75%.

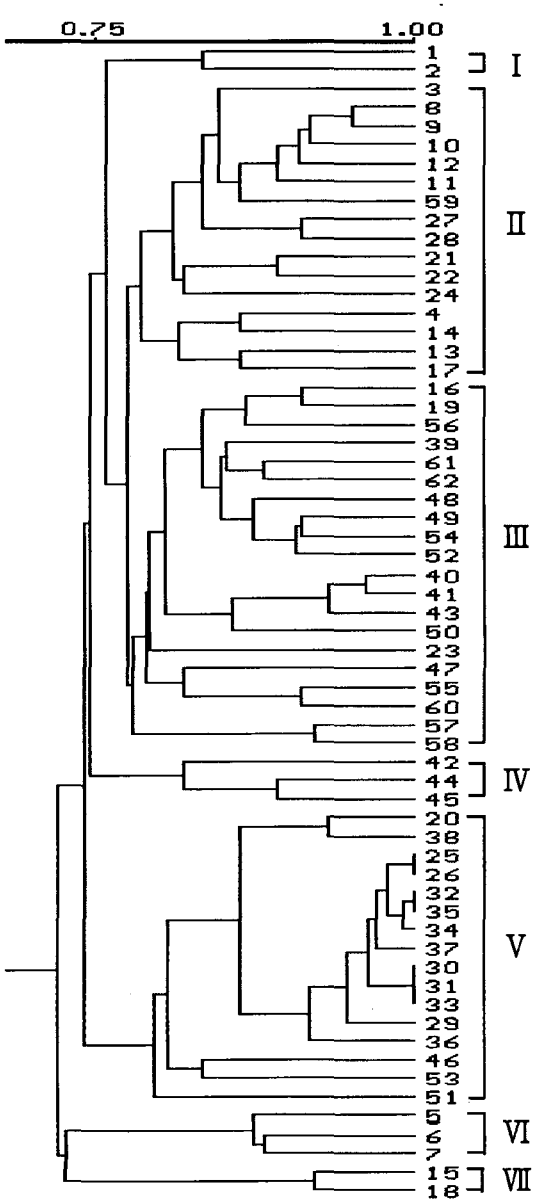

Fig. 2. Phylogenic classification of 62 accessions of *Allium* subgenus *Rhizirideum* by URP Primers.

Genetic relationship among Rhizirideum accessions

Subgenus *Rhizirideum* accessions is divided into seven groups at 0.76-similarity level (Fig. 2). Two accessions of *A. anisopodium* are separated as groupI. The species was classified in section *Rhizirideum* (Chinese Academy of Science, 1980) and in section *Anisopodia* (Fritsch, 1992). The authors follow the latter reference since the morphological characters of the species, such as leaf cross section, is quite different from the species of section *Rhizirideum*. Classification of section *Rhizirideum* still has some disagreement among references (Chinese Academy of Science, 1980; Pritsch, 1992; Hanelt, 1996; Ohri *et al.*, 1998).

In group II, relatives of Chinese chives, such as *A. austrosibiricum, A. libani, A. senescens, A. senescens* var. *minor, A. senescens* ssp. *montanum, A. nutans, A. odorum,* and *A. narcissiflorum* were clustered at 0.80-similarity level. In this group, five accessions composed of *A. libani, A. senescens* ssp. *montanum,* and *A. nutans* showed higher similarity than other species. Ulleungdobuchu, identified as *A. senescens,* has broader and thicker leaves than Pabuchu and Solipbuchu. It located more or less far from other accessions of *A. senescens*. In *A. odorum*, No. 13 and No. 14 showed high similarity, while No. 15 showed different band type. Former two accessions were tetraploid (2n=32), the latter was diploid with B-chromosome (2n=16+1B) (Buyanchimeg, 1997). The species was recognized as a synonym of *A. ramosum* and identified as *A. ramosum* var. *odorum* (Sancir, 1992). From this experiment three accessions of the species are far away from those of *A. ramosum* and *A. tuberosum*, in spite of their morphological resemblance.

Group III is composed of *A. prostratum, A. mongolicum, A. splendens, A. thungergii,* and *A. hymenorrhizum.* Two accessions of *A. prostratum* out of four belonged to this group. But the tetraploid, No. 17, was involved in group II and No. 18 was group in VII. In *A. mongolicum*, three accessions of diploid, No. 40, No. 41, and No. 43, belonged to group III, while two accessions of tetraploid, No. 42 and No. 44, were classified into group IV. This species showed higher similarities of more than 86% within same polyploids, while lower similarities of less than 72% between dif-

ferent polyploids. *A. cyaneum*, an endemic species of southern mountainous region of Korea, showed different type from other species. Its synonym is *A. taquetii* Lev. et Vnt. (Yu, 1980). Two accessions of *A. polyrhizum* were arranged in IV group and V.

In group V, cultivated Chinese chives (*A. tuberosum*), composed of 11 local collections from Korea, Taiwan, and China, showed 87% similarity. In addition, accessions collected from Ulju, Gochang and Wanju and accessions from Pyeongchang, Jeongseon and Yesan shown same band pattern within group in four primers used. The former three accessions were collected from southern Korean peninsula and latter three accessions were from middle Korea. While Muju collection, located in mountainous region, showed different band type from other local collections. *A. ramosum* and *A. tuberosum* represented high similarity of 0.91. *A. ramosum* is presumed to be an ancestor of cultivated *A. tuberosum* (Davies, 1992). Also this result corresponds with the reference of genomic DNA content (Ohri *et al.*, 1998) and dot blot hybridization with randomly amplified DNA probes (Dubouzet *et al.*, 1997). Three accessions of *A. lineare*, No.5, No.6 and No.7, showed high similarity and arranged into group VI, while one accession, No.4, represented different band pattern. The former three accessions were collected in northern Mongolia, while No. 4 was collected in high mountains of northeastern Mongolia.

Overall phylogenic data from DNA fingerprint by URP primers corresponded to classification from morphological characters. Genetic diversity in cultivated species, *A. tuberosum*, is narrower than that in wild species. Some of wild species, such as *A. prostratum*, *A. polyrhizum*, *A. odorum*, and *A. mongolicum* showed different band patterns within same species depending on polyploidy, B-chromosome, collection site and origin. This result indicates that wild *Allium* species may have different genome constitution even within same species depending on chromosome polyploidy, occurrence of B-chromosome or geographic isolation. Further studies on speciation of subgenus *Rhizirideum* is recommended. Inter-specific hybridization between Chinese chives and its relatives may broaden genetic diversity of Chinese chives. One accession of *A. polyrhizum*, No. 46, for example, can be considered as a candidate for hybridization, because it showed relatively high similarity and same ploidy level.

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